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PROMOTION OF CENTRAL NERVOUS SYSTEM REMYELINATION USING MONOCLONAL AUTOANTIBODIES

This Application is a continuation-in-part of copending Application U.S.S.N. 08/236,520, filed April 29, 1994, now issued U.S. Patent 5,591,629

GOVERNMENT SUPPORT

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BACKGROUND OF THE INVENTION

Multiple sclerosis (MS) is a chronic, frequently progressive, inflammatory central nervous system (CNS) disease characterized pathologically by primary demyelination, usually without initial axonal injury. The etiology and pathogenesis of MS are unknown. Several immunological features of MS, and its moderate association with certain major histocompatibility complex alleles, has prompted the speculation that MS is an immune-mediated disease.

An autoimmune hypothesis is supported by the experimental autoimmune (allergic) encephalomyelitis (EAE) model, where injection of certain myelin components into genetically susceptible animals leads to T cell-mediated CNS demyelination. However, specific autoantigens and pathogenic myelin-reactive T cells have not been definitively identified in the CNS of MS patients, nor is MS associated with other autoimmune diseases. An alternative hypothesis, based upon epidemiological data, is that an environmental factor, perhaps an unidentified virus, precipitates an inflammatory response in the CNS, which leads to either direct or indirect ("bystander") myelin destruction, potentially with an induce autoimmune component. This hypothesis is supported by evidence that

several naturally occurring viral infections, both in humans and animals, can cause demyelination. One commonly utilized experimental viral model is induced by Theiler's murine encephalomyelitis virus (TMEV) (Dal Canto, M.C., and Lipton, H.L., Am. J. Path., 88:497-500 (1977)).

The limited efficacy of current therapies for MS and other demyelinating diseases, has stimulated interest in novel therapies to ameliorate these diseases. However, due to the apparently complex etiopathogenesis of these diseases, potentially involving both environmental and autoimmune factors, the need still exists for an effective treatment of these demyelinating disorders.

SUMMARY OF THE INVENTION

The present invention relates to the promotion, or stimulation, or remyelination of central nervous system axons in a mammal. Specifically, the present invention relates to methods of stimulating the remyelination of central nervous system (CNS) axons using autoantibodies of the IgM subtype, or active fragments thereof, characterized by their polyreactivity, and encoded by unmutated germline genes. These monoclonals (mAbs) are referred to herein as SCH94.03, SCH 79.08, O1, O4, A2B5 and HNK-1. Of these monoclonal antibodies, O1, O4, A2B5 and HNK-1 are well-known oligodendrocyte-reactive (OL-reactive) monoclonal antibodies. See, for instance, Eisenbarth et al., Proc. Natl. Acad. Sci. USA, 76 (1979), 4913-4917, and Abo et al. J. Immunol., 127 (1981), 1024-1029). ~~The monoclonal antibodies referred to as SCH94.03 and SCH 79.08, and the corresponding hybridomas producing them, have been deposited on April 28, 1994, and February 27, 1996, respectively, under the terms of the Budapest Treaty, with the American Type Culture Collection (ATCC)~~

~~and given ATCC Accession Nos. CRL 11627 and HB12057, respectively~~.

The present invention utilizes an analysis of the Ig variable region cDNA sequences and the polyreactivity of these mAbs by ELISA to ascertain their utility in the methods described herein. Further, this work provides confirmation of the generic utility of this group of germline natural autoantibodies as effective in producing remyelination of the central nervous system.

The present invention also relates to methods of treating demyelinating diseases in mammals, such as multiple sclerosis in humans, and viral diseases of the central nervous system of humans and domestic animals, such as post-infectious encephalomyelitis, or prophylactically inhibiting the initiation or progression of demyelination in these disease states, using the monoclonal antibodies, or active fragments thereof, of this invention. This invention further relates to *in vitro* methods of producing, and stimulating the proliferation of, glial cells, such as oligodendrocytes, and the use of these glial cells to treat demyelinating diseases.

It is thus an object of the present invention to provide methods for treating demyelinating diseases in mammals, such as multiple sclerosis in humans, and viral diseases of the central nervous system of humans and domestic animals, such as post-infectious encephalomyelitis, or prophylactically inhibiting the initiation or progression of demyelination in these disease states, using the described monoclonal autoantibodies, active fragments thereof, or other natural or synthetic autoantibodies having the characteristics of mAb SCH94.03, SCH 79.08, O1, O4, A2B5 and HNK-1.

It is further an object of the present invention to provide *in vitro* methods of producing, and stimulating the proliferation of, glial cells, such as oligodendrocytes, and the use of these glial cells to treat demyelinating diseases.

BRIEF DESCRIPTION OF THE DRAWING FIGURES

Figure 1 is a graph depicting the dose-response characteristics of antibody-mediated proliferation of cells in mixed rat brain culture.

Figure 2 is a graph depicting the temporal profile of antibody-mediated proliferation of cells in mixed rat brain culture.

Figure 3A-3D shows light and electron micrographs of CNS remyelination promoted by mAb SCH94.03. (A) Light micrograph of spinal cord section from a chronically infected SJL/J mouse treated with SCH94.03 showing CNS remyelination. (B) Light micrograph of spinal cord section from a chronically infected SJL/J muse treated with a control IgM showing extensive demyelination, and the relative absence of remyelination. Inflammatory cells, including macrophages with ingested myelin debris are indicated by arrows. The asterisk indicates a representative naked axon. (C) Light micrograph of spinal cord section with normal myelin. (D) Electron micrograph of spinal cord section from an animal treated with SCH94.03 showing multiple axons with abnormally thin myelin sheaths relative to axon diameter. The star in the upper right-hand corner indicates an axon with normal myelin sheath thickness. Arrowheads point to astrocytic processes, which are intimately associated with remyelinated axons. Scale bars represent 13 μm in A-C, and 2 μm in D.

Figure 4 is a graph depicting the correlation between the change in clinical disease and morphological remyelination.

Figure 5 is a graph depicting the dose-response relationship between treatment with mAb SCH94.03 and CNS remyelination. Area of CNA remyelination (•) and percentage of lesion area with remyelination (o) in animals treated with various doses of mAb SCH94.03.

Figure 6 shows a Western blot of TMEV proteins. Lysates from infected L2 fibroblast cells were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with SCH94.03 (lane 1), SCH93.32 (lane 2), serum from susceptible mice chronically infected with TMEV (lane 3), and polyclonal rabbit anti-TMEV IgG (lane 4). Molecular weights are indicated on the left in kilodaltons (kDa). The position and identification of the major TMEV capsid proteins are indicated on the right.

Figure 7A-7D shows the immunostaining of cultured glial cells and frozen CNS tissue sections with mAb SCH94.03. Scale bars represent 15 μm.

Figure 8A-8C shows the results of SCH94.03 (Figure 8A) and control IgMs (Figure 8B and 8C) binding to protein antigens as determined by ELISA.

Figure 9 shows the results of SCH94.03 F(ab2)' binding to protein antigens as determined by ELISA.

Figure 10A-10C show the results of SCH94.03 (Figure 10A) and control IgMs (Figure 10B and 10C) binding to chemical haptens as determined by ELISA.

Figure 11 shows the alignment of the immunoglobulin light and heavy chain variable region sequences of SCH94.03 and control IgM, CH12, and germline Ig gene segments.

Figure 12 shows the nucleotide and deduced amino acid sequences of V_H, D and J_H regions encoding O1, compared with the unrearranged V_H segment transcript A1 and A4, and the JH germline gene (SEQ. ID 1). Dashed lines indicate identity with unrearranged V_H segment transcript A1 and A4. Underline indicates identity with germline AP2 gene family (DSP2.3, 2.4, 2.6). Amino acids are represented by the single-letter code. CDR represents the complementarity determining region. This sequence has been assigned the GenBank TM/EMBL Data Bank Accession number L41877.

Figure 13 shows the nucleotide and deduced amino acid sequences of V_H, D and J_H regions encoding O4 and HNK-1 (SEQ. ID 2), compared with those reported for germline gene V_H101 and J_H, and for natural autoantibody D23. Dashed lines indicate identity with V_H101 and J_H4. Underline indicates identity with germline DFL16.1. Amino acids are represented by the single-letter code. CDR represents the complementarity determining region. These sequences have been assigned the GenBank TM/EMBL Data Bank Accession Numbers L41878 (O4) and L41876 (HNK-a).

Figure 14 shows the nucleotide and deduced amino acid sequences of V_H, D and J_H regions encoding A2B5 (SEQ. ID 3), compared with those reported for germline gene V1 and J_H3 germline gene. Dashed lines indicate identity with germline gene V1 and J_H3. Underline indicates identity with germline DFL16.2. Amino acids are represented by the single-letter code. CDR represents the complementarity determining region. This sequence has

been assigned the GenBank TM/EMBL Data Bank Accession Number L41874.

Figure 15 shows the nucleotide and deduced amino acid sequences of V_H and J_H regions encoding O1 and O4 (SEQ. ID 4), compared with those reported for myeloma MOPC21, for natural autoantibody E7 and for 3_x2 germline gene. Dashed lines indicate identity with MOPC21 and germline gene J_H2 (N, undetermined nucleotide). Amino acids are represented by the single-letter code. CDR represents the complementarity determining region. These sequence have been assigned the GenBank TM/EMBL Data Bank Accession Numbers L41879 (O1) and L41881 (O4).

Figure 16 shows the nucleotide and deduced amino acid sequences of V_H and J_H regions encoding HNK-1 (SEQ.ID 5), compared with those reported for germline V_H41, myeloma MOPC21, and J_H2. Dashed lines indicate identity with germline genes. Amino acids are represented by the single-letter code. CDR represents the complementarity determining region. This sequence has been assigned the GenBank TM/EMBL Data Bank Accession Number L41880.

Figure 17 shows the nucleotide and deduced amino acid sequences of V_H and J_H regions encoding A2B5 (SEQ ID 6). Dashed lines indicate identity with germline J_H. Amino acids are represented by the single-letter code. CDR represents the complementarity determining region. This sequence has been assigned the GenBank TM/EMBL Data Bank Accession Number L41875.

Figure 18 is a graph showing the reactivity of O1, O4, A2B5 and control (TEPC183 and XXMEN-OES)IgMκ mAbs by direct ELISA.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the promotion, or stimulation, or remyelination of central nervous system axons in a mammal. Specifically, the present invention relates to methods of stimulating (the remyelination of central nervous system (CNS) axons using a monoclonal autoantibody of the IgM subtype) or an active fragment thereof, characterized by its polyreactivity and encoded by unmutated germline genes, or a natural or synthetic analog thereof.

As used herein, the term "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term is intended to encompass polyclonal, monoclonal, and chimeric antibodies, the last mentioned described in further detail in U.S. Patent Nos. 4,816,397 and 4,816,567. Such antibodies include both polyclonal and monoclonal antibodies prepared by known generic techniques, as well as bi-specific (chimeric) antibodies, and antibodies including other functionalities suiting them for additional diagnostic use conjunctive with their capability of modulating ~ activity stimulating the remyelenation of CNS axons. An "antibody combining site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen. The phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule. Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the

paratope, including those portions know in the art as Fab, Fab', $F(ab')_2$ and F(v).

Fab and $F(ab')_2$ portions of antibody molecules are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. See for example, U.S. Patent No. 4,342,566 to Theofilopolous et al. Fab' antibody molecule portions are also well-known and are produced from $F(ab')_2$ portions followed by reduction of the disulfide bonds linking the two heavy chains portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules is preferred herein.

The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; e.g., a bispecific (chimeric) monoclonal antibody.

The present invention also relates to methods of treating demyelinating diseases in mammals, such as multiple sclerosis in humans, and viral diseases of the central nervous system of humans and domestic animals, such as post-infectious encephalomyelitis, using the SCH 94.03, SCH 79.08, O1, O4, A2B5 and HNK-1 monoclonal antibodies, an active fragment thereof, or a natural or synthetic autoantibody having the characteristics thereof. Methods of prophylactic treatment using these mAb, active fragments thereof, or other natural or synthetic

autoantibodies having the same characteristics, to inhibit the initiation or progression demyelinating diseases are also encompassed by this invention.

Oligodendrocytes (OLs), the myelin-forming cells of the central nervous system (CNS), originate as neuroectodermal cells of the subventricular zones, and then migrate and mature to produce myelin. The sequential development of OLs is identified by well-characterized differentiation stage-specific markers. Proliferative and migratory bipolar precursors, designated oligodendrocyte/type-3 astrocyte (O-2A) progenitors, are identified by monoclonal antibodies (mAbs) anti-GD_3 and A2B5 [Eisenbarth et al., Proc. Natl. Acad. Sci. USA, 76 (1979), 4913-4917]. The next developmental stage, characterized by multipolar, postmigratory, and proliferative cells, is recognized by mAb O4 [Gard et al., Neuron, 5 (1990), 615-625; Sommer et al., Dev. Biol., 83 (1981), 311-327]. Further development is defined by the cell surface expression of galactocerebroside, recognized by mAb O1 [Schachner, J. Neurochem., 39 (1982), 1-8; Sommer et al., *supra*], and by the expression of 2′,3′-cyclic nucleotide 3′-phosphohydrolase. The most mature cells express terminal differentiation markers such as myelin basic protein and proteolipid protein.

The mAbs (A2B5, O1, and O4) used to characterize the stages of OL development were made by immunizing BALB/c mice with chicken embryo retina cells or homogenate of bovine corpus callosum [Eisenbarth et al., *supra*; Sommer et al., *supra*]. A2B5 recognizes not only O-2A progenitors but also neurons and reacts with cell surface ganglioside GQ1c [Kasai et al., Brain Res., 277 (1983), 155-158] and other gangliosides [Fredman et al., Arch. Biochem. Biophys., 233 (1984), 661-666]. O4 reacts with sulfatide, seminolipid and cholesterol [Bansal et al.,

J. Neurosci. Res., 24 (1989), 548-557], whereas O1 reacts with galactocerebroside, monogalactosyl-diglyceride and psychosine [Bansal et al., *supra*]. These mAbs belong to the IgM immunoglobulin (Ig) subclass and recognize cytoplasmic structures as well as the surface antigens of OLs [Eisenbarth et al., *supra*; Sommer et al., *supra*]. Mouse mAb HNK-1 (anti-Leu-7), made by immunizing BALB/c mice with the membrane suspension of HSB-2 T lymphoblastoid cells, was first reported as a marker for natural killer cells [Abo et al., J. Immunol., 127 (1981), 1024-1029]. Later, HNK-1 was shown to share antigenic determinants with the nervous system [Schuller-Petrovic et al., Nature, 306 (1983), 179-181]. The carbohydrate epitope on myelin-associated glycoprotein, found in both central and peripheral myelin sheaths, was shown to be a principal antigen of nervous tissue the reacted with HNK-1 [McGarry et al., Nature, 306 (1983), 376-378]. However, other glycoproteins in nervous tissue react with this mAb, some of which are important in embryogenesis, differentiation, and myelination [Keilhauer et al., Nature, 316 (1985), 728-730; Kruse et al., Nature, 311 (1984), 153-155; Kruse et al., Nature, 316 (1985), 146-148; McGarry et al., J. Neuroimmunol., 10 (1985), 101-114]. Of interest, HNK-1 also reacts with cytoplasmic structures and belongs to the IgM Ig subclass.

A monoclonal antibody, disclosed and claimed in copending parent application U. S. S. N. 08/236,520, filed April 29, 1994, and designated SCH94.03, was found to promotes CNS remyelination in mice infected chronically with Theiler's murine encephalomyelitis virus (TMEV) [Miller et al., J. Neurosci., 14 (1994), 6230-6238]. SCH94.03 belongs to the IgM(κ) Ig subclass and recognizes an unknown surface antigen on OLs, but cytoplasmic antigens in all cells (Asakura et al., Molecular Brain Research, in press). The polyreactivity of SCH94.03 by ELISA, and

the unmutated Ig variable region germline sequences indicated that SCH94.03 is a natural autoantibody [Miller et al., J. Neurosci., 14 (1994), 6230-6238]. A close study of SCH94.03, and comparison thereof with well-known OL-reactive mAbs A2B5, O1, O4, and HNK-1 raised the possibility that these are natural autoantibodies. A subsequent analysis of the Ig variable region cDNA sequences and the polyreactivity of these mAbs by ELISA confirmed that this is a generic group of natural autoantibodies having similar utilities.

The antigen reactivity of the monoclonal antibody, IgM monoclonal antibody referred to herein as SCH 94.03 (also referred to herein as SCH94.32) and SCH 79.08 (both prepared from a mammal immunized with spinal cord homogenate from a normal mammal (i.e., uninfected with any demyelinating disease)), have been characterized and described in the aforesaid parent Application U.S.S.N. 08/236,520, filed April 29, 1994, whose teachings are incorporated herein by reference, using several biochemical and molecular assays, including immunohistochemistry, immunocytochemistry, Western blotting, solid-phase enzyme-linked immunosorbant assays (ELISA), and Ig variable region sequencing. The hybridomas producing monoclonal antibody SCH 94.03 and SCH 79.08 have been deposited on April 28, 1994, and February 27, 1996, respectively, under the terms of the Budapest Treaty, with the American Type Culture Collection (ATCC) and have been given ATCC Accession Nos. CRL 11627 and HB12057, respectively. All restrictions upon the availability of the deposit material will be irrevocably removed upon granting of a patent.

Natural or physiologic autoantibodies are present normally in serum, are polyreactive, are frequently of the IgM subtype, and are encoded by unmutated germline

genes. By sequencing immunoglobulin (Ig) cDNAs of the oligodendrocyte-reactive O1, O4, A2B5, and HNK-1 IgM κ monoclonal antibodies and comparing these with published germline sequences, it was determined that these were natural autoantibodies. O1 V_H was identical with unrearranged V_H segment transcript A1 and A4, O4 V_H had three and HNK-1 V_H had six nucleotide differences from germline V_H101 in the V_H coding region. The D segment of O1 was derived from germline SP2 gene family, J_H4, whereas O1 J_H was encoded by germline J_H1 with one silent nucleotide change. O1 and O4 light chains were identical with myeloma MOPC21 except for one silent nucleotide change. HNK-1 $V_κ$ was identical with germline $V_κ$41 except for two silent nucleotide changes. O1 $J_κ$, O4$J_κ$ and HNK $J_κ$ were encoded by unmutated germline $J_κ$2. In contrast, A2B5 V_H showed seven nucleotide differences from germline V1, whereas no germline sequence encoding A2B5 $V_κ$ was identified. O1 and O4, but not A2B5 were polyreactive against multiple antigens by direct ELISA. Therefore, O1, O4 and HNK-1 Igs are encoded by germline genes, and have the genotype and phenotype of natural autoantibodies.

Selection of SCH mAbs to promote CNS remyelination

A panel of monoclonal antibodies (mAbs) derived from splenocytes of uninfected SJL/J mice injected with SCH was constructed as described in detail in Example 1. After the initial fusion and cloning, 2 of the 95 wells with viable Ig-secreting hybridomas contained mAb with significant binding to SCH as demonstrated by ELISA. Hybridoma cells from these two wells, called the 79 and 94 series, were subcloned by limiting dilution and screened again for binding to SCH by ELISA. For the 79 series hybridomas, 14 out of 49 clones were positive by SCH ELISA, while for the 94 series, 17 out of 32 were positive for binding to SCH. Based upon the ELISA data,

two 79 series hybridomas (SCH79.08 and SCH79.27), both of which also reacted with myelin basic protein (MBP) by ELISA, and three 94 series hybridomas (SCH94.03, SCH94.11, and SCH94.32), none of which reacted with MBP, were chosen for ascites production and *in vivo* transfer experiments.

<u>mAbs Promote Proliferation of Glial Cells</u>

As described in Example 2, the mAbs were tested for their ability to promote proliferation of glial cells *in vitro*.

The dose-response characteristic of antibody-mediated proliferation were then examined. As shown in Figure 1, maximal stimulation with 94.03 was seen at 100 ng/ml. Control myeloma IgMs MOPC 104E and TEPC 183 (data not shown) also stimulated the mixed rat brain cultures to proliferate. However, the maximal effect was seen at a 10-fold higher concentration than that seen with the mAbs.

The temporal profile of antibody-mediated proliferation was also examined as shown in Figure 2. On day 8, after culture initiation, 100 ng/ml antibody was added to the cultures (time 0). Cells were harvested at 24 hour intervals; [^{3}H]thymidine was present for the final 24 hours of culture to measure the total proliferation during the interval. The maximal stimulation with 94.03 was seen at 72 hours after antibody addition. Similar results were obtained with 94.32. None of the isotype control antibodies showed any significant proliferation throughout the 120 hours of culture. These data demonstrates that both mAbs 94.32 and 94.03 induce proliferation of glial cells of mixed rat brain culture. This proliferation is maximal at an antibody concentration of 100 ng/ml and a culture period of 72 hours after antibody addition.

<u>CNS Remyelination Promoted by mAbs SCH94.033 and SCH94.32</u>
As described in Example 3, SJL/J mice chronically infected with TMEV were treated with a total mAb dose of 0.5 mg iv or 5.0 mg ip divided into twice weekly doses for 4-5 weeks. CNS remyelination was measured by a quantitative morphological assessment on ten spinal cord cross-sections from each mouse. The criterion for CNS remyelination was abnormally thin myelin sheaths relative to axonal diameter. The data are composite of six experiments and are presented as the ± SEM, where n indicates the number of mice. Statistical comparisons for remyelination data were made with the cumulative values from both IgM and buffer only controls using a modified rank sum test. The number of demyelinated lesions and the area of demyelination were not significantly different between treatment groups assessed by a one-way ANOVA. For control IgMs, myelomas MOPC 104E and ABPC 22 (both from Sigma), and TB5-1, an anti-mycobacteria mAb, were used.

SJL/J mice chronically infected with TMEV and treated with either mAb SCH94.03 or SCH94.32 showed significantly greater CNS remyelination than animals treated with either isotype-matched control mAb or buffer only (Table 1).

TABLE 1.

Monoclonal antibodies SCH94.03 and SCH94.32 promote CNS remyelination							
Treatment	n	Number of Demylination (lesions)	Number of Remyelinated Lesions	p-value	Area of Remyelination (mm^2)	Area of Lesion (mm^2)	Area Remyelination /area Lesion (%)
SCH94.03	12	25.6 ± 2.6	12.8 ± 2.6	<0.0025	0.35 ± 0.09	1.09 ± 0.19	28.9 ± 3.8
SCH94.32	12	24.9 P ± 2.8	12.3 ± 2.3	<0.0001	0.42 ± 0.11	1.46 ± 0.21	26.7 ± 4.2
IgM control	13	29.9 ± 2.0	6.7 ± 1.2	--	0.11 ± 0.02	1.70 ± 0.28	7.7 ± 1.8
Buffer only	11	27.7 ± 2.7	5.1 ± 1.1	--	0.06 ± 0.01	1.11 ± 0.29	6.5 ± 1.2

Remyelination was seen with either iv or ip injections. SCH94.03- or SCH94.32-treated animals had approximately 2-3- fold more remyelinated lesions, and a 3-4-fold larger total area of CNS remyelination than control animals. When a cumulative statistical comparison was made using these two parameters of therapeutic effectiveness, the CNS remyelination induced by mAbs SCH94.03 and SCH94.32 was highly significant ($p < 0.005$; Table 2). In a chronic progressive disease like TMEV infection, the extent of CNS repair is a direct function of the extent of CNS damage. Both the number and area of CNS lesions were not different between treatment groups, indicating similar disease severity (Table 1). When CNS remyelination was expressed as the percentage of lesion area showing remyelination, approximately one-third of the cumulative demyelinated lesion area shown CNS remyelination in mice treated with either mAb SCH94.03 or SCH94.32 (Table 1).

Similar results were obtained using Schh 79.08 (Results shown in Table 2) and for O1, O4, A2B5 and HNK-1 (Results shown in Table 3).

Table 2. Enhancement of CNS remyelination by SCH79.08

Treatment	No. of Mice	Area of white matter (mm^2)	Area of CNS-type remyelination (mm^2)	Area of demyelinated lesion (mm^2)	Area of CNS-type remyelination/ area of lesions (%)
SCH79.08	15	8.42 ± 0.33	0.20 ± 0.05	1.01 ± 0.16	20.2 ± 4.7
PBS	6	8.89 ± 0.26	0.03 ± 0.01	1.01 ± 0.21	2.4 ± 0.8

Values represent the mean ± SEM.

Statistics byf student t-test comparing area of CNS-type remyelination/area of lesions revealed $p < 0.05$.

PBS: phosphate buffered saline.

Table 3. Enhancement of CNS remyelination by oligodendrocyte-reactive monoclonal antibodies

Treatment	No. of mice	Area of White matter (mm^2)	Area of CNS-type remyelination (mm^2)	Area of demyelinated lesion (mm^2)	Area of CNS-type remyelination/ area of lesions (%)
O1	6	7.57 ± 0.52	0.14 ± 0.04	0.53 ± 0.10	24.8 ± 6.2*
O4	7	8.01 ± 0.15	0.17 ± 0.04	0.84 ± 0.10	20.4 ± 4.2*
A2B5	7	7.28 ± 0.38	0.18 ± 0.05	0.70 ± 0.17	24.6 ± 4.6*
HNK-1	7	7.16 ± 0.38	0.15 ± 0.03	0.78 ± 0.10	20.6 ± 2.8*
PBS	6	7.46 ± 0.70	0.05 ± 0.02	0.51 ± 0.14	8.0 ± 2.2

Values represent the mean ± SEM.

Statistics by student t-test comparing area of CNS-type remyelination/area of lesions revealed *:$p<0.05$, **:$p<0.01$.

PBS: phosphate buffered saline

Morphology of CNS Remyelination

CNS remyelination was readily identified morphologically both by light and electron microscopy (Figure 3A-3D). Figure 3A shows a remyelinated lesion from an animal treated with SCH94.03. The majority of axons in the lesion show morphologic evidence of repair, with abnormally thin myelin sheaths relative to axonal diameter (Ludwin, S.K. "Remyelination in the central nervous system of the mouse," In: THE PATHOLOGY OF THE MYELINATED AXON (Adachi M, Hirano A, Aronson SM eds), pp 49-79, Tokyo: Igaku-Shoin Ltd. (1985)). For comparison, Figure 3B shows a demyelinated lesion, with minimal remyelination, whereas Figure 3C is an area of normal myelin, with thickly myelinated axons. Within remyelinated lesions (Figure 3A), there were 15.3 ± 1.0 (mean ± SEM) myelinated axons per 100 μm^2, compared to only 1.1 ± 0.2 myelinated axons per 100 μm^2 in demyelinated lesions (Figure 3B). Figure 3C shows a light micrograph of spinal cord section with normal myelin. By electron microscopy, CNS remyelination was especially evident (Figure 3D). Almost every axon in the field has evidence of new myelin formation, although the degree of remyelination (i.e., myelin thickness) is variable between individual axons, suggesting different stages of the repair process. The ratio of myelin thickness to axonal diameter was 0.08 ± 0.01 (mean ± SEM; n = 25 axons) for remyelinated axons compared to 0.21 ± 0.01 (n = 34 axons) for normally myelinated axons.

Correlation Between Clinical Disease and Morphological Remyelination

The correlation of morphological remyelination with clinical signs of disease improvement was assessed as described in Example 3. At each treatment injection, mice were assessed clinically as described in Example 3. The change in clinical score was correlated with the

percentage of lesion area showing remyelination (Figure 4). Morphological remyelination is represented as the percentage of lesion area showing CNS remyelination. A change in clinical score of 0 represent stable disease over the treatment period (4-5 weeks), whereas a positive change indicates worsening of clinical disease, and a negative change indicates improvement. Data represent individual animals from all treatment groups. A positive change in clinical score indicates worsening of disease. Using data from all treatment groups, the change in clinical score showed a moderate but significant negative correlation ($R=-0.40$; $p < 0.04$) with the percentage of lesion area showing remyelination. Although few animals actually improved clinically (Δ clinical score < 0), animals with an increase in disease severity (Δ clinical score > 0) tended to have less morphological remyelination, while animals that remained stable clinically (Δ clinical score $= 0$) showed the most remyelination. A similar negative correlation was obtained when the other quantitative measures of remyelination were used (the number of remyelinated lesions and the area of remyelination) as shown in Table 1. These data demonstrate that remyelination quantitated by morphology is associated with slowing of clinical disease progression.

Titration of mAb SCH94.03 Dose and CNS Remyelination

For the initial treatment experiments, a total mAb dose of 25 mg/kg for intravenous (iv) injections and 250 mg/kg for intraperitoneal (ip) injection was empirically chosen. To assess the dose-response characteristics, and to determine the minimal amount of mAb needed to promote remyelination, chronically-infected mice were treated with various ip doses of SCH94.03. Remyelination was quantitated as described for Table 1. Data are the mean values of 4-5 animals per mAb dose, with the final cumulative dose indicated on the graph. SEM averaged 35%

of the mean. There was no statistical difference assessed by one-way ANOVA in the number of demyelinated lesions or the area of demyelination between treatment groups, indicated similar extent of disease in all animals. The number of demyelinated lesions and area of lesions were 33.2 ± 7.5 and 1.25 ± 0.43 for the 1000 μg group, 31.8 ±8 and 1.11 ± 0.31 for the 100 μg group, 23.8 ± 3.4 and 0.54 ± 0.14 for the 10 μg group, and 20.0 ± 6.5 and 0.74 ± 0..20 for the buffer only group (represented as the 0 dose point on the graph). Animals treated with 100 μg control IgM (MOPC 104E) had remyelination scores similar to control animals treated with buffer only. The positive correlation between the dose of mAb SCH94.03 and CNS remyelination was especially striking when the severity of CNS disease was taken into account. When CNS repair was expressed as the percentage of lesion area showing remyelination, mice treated with a total dose of 1000, 100, or 10 μg of SCH94.03 had 6-, 5-, and 4-fold more remyelination than control animals, respectively (Figure 5). Mice given as little as 10 μg of SCH94.03 ip (0.5 mg/kg) showed evidence of enhanced CNS remyelination. These data indicated that mAb SCH94.03 and CNS remyelination had a positive does-response relationship, and that very small quantities of mAb were needed to promote myelin repair.

Antigen Specificity of SCH94.03 and SCH94.32

Although mAbs SCH94.03 and SCH94.32 were generated from splenocytes of uninfected mice, and screened against SCH from uninfected mice, it was directly assessed whether either mAb could react with TMEV capsid proteins or inhibit viral infectivity *in vitro*. By Western blotting (Figure 6), SCH94.03 and SCH94.32 did not react with any TMEV proteins recognized by either serum from chronically infected mice or polyclonal IgG from rabbits injected with purified TMEV (Rodriguez, et al., Ann. Neurol., 13:426-433 (1983)). Western blot of lysates from control

mock infected L2 cells showed single bands with the serum from chronically infected animals and the polyclonal rabbit anti-TMEV IgG at 32 and 43 kDa, respectively, but no reactivity with SCH94.03 or SCH94.32.

In addition, no significant inhibition of TMEV infectivity *in vitro* with up to 5 μg/ml of either SCH94.03 or SCH94.32, was observed under assay conditions where 50% neutralization was observed with a 1:34,000 dilution of serum from chronically infected animals. These results indicated that the therapeutic effect of SCH94.03 and SCH94.32 was not due to direct inhibition of the virus.

To initially characterize the antigens recognized by mAbs SCH94.03 and SCH94.32, various cell lines derived from glial (rat C6, mouse G26-20, human U373MG and U87MG), neural (human neuroblastoma), fibroblast (mouse L and 3T3), epithelial (human SCC-9 carcinoma), and lymphocytic (mouse CTLL2) origin were stained. Both mAbs stained internal antigens of all cell lines tested, which indicated that certain antigens recognized by these mAbs were not restricted to unique cell types *in vitro*. Based on the hypothesis that the therapeutic effect of SCH94.03 and SCH94.32 was due to a CNS-specific interaction, the immunostaining of cultured cells by SCH94.03 and SCH94.32 using the rat glial cell line 5.5B8 was further investigated. This immortalized glial cell line has phenotypic characteristics of both oligodendrocytes and astrocytes, with expression of MBP and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP), and low, but detectable, expression of glial fibrillary acidic protein (GFAP) and the lipids or proteins recognized by the mAbs A2B5 and 04 (Bozyczko, et al., Ann. NY Acad. Sci., 605:350-353 (1990)). SCH94.03 and SCH94.32 recognized both a surface and cytoplasmic determinant on 5.5B8 cells. The surface staining was most prominent on small

cells which lay on top of a layer of flat, morphologically differentiated cells (Figure 7A). Surface staining was confirmed by flow cytometry on live cells. When the cell membrane was permeabilized by dehydration or brief treatment with a non-ionic detergent to expose internal antigens, the staining pattern was altered considerably (Figure 7B). The cytoplasmic staining was filamentous, with a dense perinuclear network that extended out into the cell processes. This pattern closely resembled the staining pattern of the intermediate filament cytoskeletal protein vimentin. These data indicated that SCH94.03 and SCH94.32 recognized antigens that were not restricted to cells derived from the nervous system, but that they did recognize both surface and cytoplasmic determinants on glial cells.

Immunohistochemical staining of frozen mouse, rat, and human tissue confirmed that SCH94.03 and SCH94.32 were not CNS-specific mAbs, but rather showed multi-organ reactivity. Both mAbs immunostained all major organs examined, including the brain, spinal cord, optic nerve, heart, liver, kidney, stomach, and small intestine and skeletal muscle. However, not all cells within an organ stained, suggesting *in situ* cytological specificity. Within the CNS, SCH94.03 and SCH94.32 stained predominately blood vessels, ependymal cells, and stellate-shaped cells with the morphological features of glial cells, which were enriched in neonatal cerebellar, periventricular, and brain stem white matter (Figure 7C), and both neonatal and adult optic nerve. Similar glial cells positive for SCH94.03 and SCH94.32 were found in autopsied human brain tissue, especially at the gray-white matter junction (Figure 7D). Identical immunostaining results were obtained with mAb SCH94.32. Immunostaining with a control IgM (MOPC 104E) was

negative for all samples and tissue structures which immunostained with SCH94.03 and SCH94.32.

The identification and characterization of an entire family of autoantibodies, referred to as "natural" or "physiological" autoantibodies, has influenced traditional view of autoimmunity and self-reactivity. The natural autoantibodies that have been studied extensively are typically IgMs, although other isotypes have been identified, are reactive toward a wide range of antigens, including cytoskeletal proteins, surface proteins, nucleic acids, phospholipids, bacterial antigens such as lipopolysaccharides, and various chemical haptens (reviewed by Avrameas and Ternynck, Mol. Immunol., 30:1133-1142 (1993)). Natural autoantibodies share extensive idiotypic cross-reactivity or "connectivity", which includes expression of similar idiotypes, some of which are expressed by pathogenic autoantibodies, as well as reactivity toward common idiotypes expressed on other antibodies. Molecular analysis has shown that natural autoantibodies are typically encoded by unmutated germline immunoglobulin (Ig) genes, with few if any somatic mutations, and therefore represent a substantial fraction of the Ig repertoire, especially in neonatal animals which have not had extensive exogenous antigen exposure.

The function of natural autoantibodies remains enigmatic. Several hypotheses have been proposed based upon their biochemical and molecular characteristics. These include: (1) clearance of senescent or damage tissue, (2) providing a first line of immunological defense in the lag period between pathogen exposure and an Ag-specific immune response, (3) masking autoantigens from a potentially pathogenic autoimmune response, (4) immunomodulation, including shaping of the neonatal immune repertoire via an idiotypic network, and (5)

participation in the positive selection of B cells in the bone marrow, similar to the process proposed for T cells in the thymus.

The hypothesis that antibodies SCH94.03 and SCH94.32 were natural autoantibodies was tested. To characterize the antigen reactivities of SCH94.03 and SCH94.32, several biochemical and molecular assays, including immunohistochemistry and immunocytochemistry, Western blotting, solid-phase enzyme-linked immunosorbant assays (ELISA), and Ig variable region sequencing, were used. As described below, for all biochemical assays, SCH94.03 and SCH94.32 were indistinguishable. In addition, SCH94.03 and SCH94.32 had identical Ig variable region sequences, which confirmed that they were the same mAb. Further details of these characterizing studies are reported in Asakura et al., J. Neuroscience Res. (1996) 43, pp 273-281, which dislcosure is incorporated herein by reference.

A potential mechanism whereby SCH94.03 could stimulate remyelination in the central nervous system would be to stimulate the proliferation and/or differentiation of cells involved in myelinogenesis, primarily oligodendrocytes or their immature precursors. Thus, it was tested whether SCH94.03 stained the surface of various cells. Using immortalized cells, it was determined that SCH94.03 stained two glial cells lines, 5.5B8 (Figure 7A) and 20.2E11, but did not stain the surface of several other glial cells lines (10.IA3, 20.2A40, C6, G26-20), a neuroblastoma cell line (B104), two fibroblast lines (L2, Cos-1), or two myoblastomas (G8, L6). Similar results were obtained with cells isolated from animal tissues and grown in culture. SCH94.03 stained the surface of oligodendrocytes, but not astrocytes, microglia, Schwann cells, myoblasts, or fibroblasts.

The reactivity of SCH94.03 with proteins from glial and lymphoid cell lines, and tissue lysates from brain, liver, and intestine by Western blotting was also assessed. SCH94.03 reacted with multiple bands from all cells and tissues examined, with prominent reactivity towards bands at 50, 95, 120, and >200 kDa. The exact identity of these protein bands has not been determined.

The activity of SCH94.03 with several purified protein self-antigens by solid-phase ELISA was determined. (Figure 8A-8C). SCH94.03 showed strong reactivity toward the RBC antigen spectrin, but also showed consistent reactivity toward hemoglobin, actin, tublin, and vimentin, and thyroglobulin, although to a lesser qualitative degree than toward spectrin. No reactivity was observed with myosin, transferrin, albumin, lysozyme, or myelin basic protein under our assay conditions. Six other monoclonal or myeloma IgM controls XXMEN-OE5 (Figure 8B), A2B5, MOPC104E, TEPC183, 01, and CH12 (Figure 8C), were also tested, and no reactivity with any of the antigens tested was observed.

To confirm the monoclonality of SCH94.03, 18 subclones of SCH94.03 (9 each from SCH94.03 and SCH94.32 parents) were tested for polyreactivity by solid-phase ELISA. All 18 subclones showed identical reactivity patterns with the panel of protein antigens as the parent SCH94.03. To further support the conclusion that the polyreactivity of SCH94.03 was via its Fab region, we generated $F(ab)_2'$ fragments and assessed their reactivity with the protein antigens by ELISA (Figure 9). SCH94.03 $F(ab)_2'$ fragments showed similar polyreactivity as the whole IgM molecule.

A panel of chemical haptens coupled to bovine serum albumin (BSA) was constructed and used to assess SCH94.03 reactivity by solid-phase ELISA (Figure 10A-10C). SCH94.03 showed strong reactivity toward fluorescein (FL)

and 4-hydroxy-3-nitrophenyl acetic acid (NP), moderate reactivity toward phenyloxazolone (PhOx), and weak reactivity toward 2, 4, 6-trinitrophenyl (TNP) and *p*-azophenylarsonic acid (Ars). No reactivity with *p*-azophenyltrimethylammonium (TMA), *p*-azophenylphosphorylcholine (PC), or the carrier protein BSA was detected. Control IgMs (Figure 10B and 10C) showed no significant binding to any of the haptens tested, with the exceptions of CH12 reactivity with TMA, which has been previously reported, and A2B5 reactivity with NP.

It was further investigated whether the Ig light (L) and heavy (H) chains of SCH94.03 were encoded by germline Ig genes (Figure 11). The light chain variable (V_L) and joining (J_L) region nucleotide sequences from SCH94.03 had 99.4% identity with the previously published sequences of the germline Vκ10 and Jκ1 genes, with only two silent changes at the 3′ end of both the V_L and J_L regions. The SCH94.03 V_H region nucleotide sequence was identical to the previously published germline V_H23 sequence, the J_H region sequence differed from the published germline J_H2 sequence by one nucleotide, at the 5′ end of the J region, and the diversity (D) region contained 15 contiguous nucleotides derived from the germline DFL16.1 gene. There were 8 nucleotides in the V-D junction, and 1 in the D-J junction, which did not correspond to any known germline V or D region genes, and probably represent noncoded (N) nucleotides inserted by the enzyme terminal deoxynucleotide transferase during V-D-J recombination. The only changes from the germline genes in the heavy chain of SCH4.03 occurred at either the V-D or D-J junction, and therefore could represent either N nucleotides or the result of imprecise joining, rather than somatic mutations. In addition, both the light and heavy chain variable regions of SCH94.03 showed extensive

sequence similarity with the IgM produced by the B-cell lymphoma CH12 (Figure 11).

These antigen reactivity results suggest that SCH94.03 is a natural autoantibody. Although this conclusion does not readily present a mechanism as to how SCH94.03 stimulates remyelination in the central nervous system, it does suggest an important physiological function of natural autoantibodies. Autoantibodies that are produced either during normal physiology, or in response to tissue damage and the subsequent release of previously sequestered antigens, might actively participate to promote repair in the damaged tissue. In line with previously proposed functions of natural autoantibodies, this active participation might be to facilitate removal of damaged tissue, mask autoantigens thereby preventing vigorous pathogenic autoimmune response, modulate the immune response which actually resulted in the tissue destruction, thereby allowing normal endogenous tissue repair to occur, or directly stimulate cells involved in the repair process.

Thus, as a result of the work described herein, it is now demonstrated that an autoantibody generated and screened for its autoantigen-binding capability, also promotes CNS remyelination. Mice chronically infected with TMEV and treated either intravenously (iv) or intraperitoneally (ip) with IgM mAbs from hybridomas SCH94-03 or SCH94.32 had significantly more CNS repair than control animals, measured by a detailed quantitative morphological assessment of CNS remyelination. Moreover, preliminary data suggest that the autoantibody, SCH94.03 is also effective in preventing clinical relapses in mammals afflicted with experimental autoimmune encephalomyelitis (EAE).

Clinical disease in SJL/J mice with established R-EAE after treatment with SCH94.03. R-EAE was induced in SJL/J mice through adoptive transfer of MBP peptide (91-103)-specific T cells and treatment was initiated with monoclonal autoantibody SCH94.03, control IgM, or PBS after recovery from the initial episode of clinical disease. Both the initial clinical disease peak and severity were similar between treatment groups (Table 4). However, treatment with SCH94.03 reduced the percentage of mice with a first clinical relapse by half compared to mice treated with control IgM or PBS, and prolonged relapse onset by 6 days in those mice that did have a clinical relapse. When only mice with severe initial clinical disease (score ≥ 3) were analyzed, 10 of 12 mice (83%) treated with control IgM or PBS had a first relapse compared to only 3 of 9 mice (33%) treated with SCH94.03 ($P < 0.04$ using a Fisher exact test), indicating that SCH94.03 was effective regardless of initial disease severity. In addition, 4 mice treated with control IgM or PBSD had a second clinical relapse, whereas no mouse treated with SCH94.03 had more than one relapse, although this difference was not statistically significant because of the few mice with a second relapse prior to sacrifice.

Spinal cord pathology in SJL/J mice with established R-EAE after treatment with SCH94.03. Treatment with SCH94.03 also improved pathological disease in established R-EAE. Consistent with the reduction in clinical disease, treatment with SCH94.03d reduced by 40% both demyelination and meningeal inflammation in the spinal cords of SJL/J mice with R-EAE (Table 5). Demyelinated lesions in mice treated with SCH94.03 were typically smaller in size with fewer inflammatory cells than mice treated with control IgM or PBS. The majority of demyelinated lesions were located in the dorsal columns in mice treated with SCH94.03 (57.0 ± 5.4%; mean ± SEM) and control IgM or PBS (51.5 ± 4.8%; $P > 0.4$ using

a Student's *t* test). The remainder of the demyelinated lesions in mice treated with SH94.03 or control IgM or PBS were distributed between posterolateral (12.0 ± 2.9% and 11.0 ± 2.0%, respectively), anterolateral (14.3 ± 3.0% and 20.3 ± 2.5%), and ventral (14.9 ± 17.1 ± 1.6%) columns ($P > 0.1$ for all).

To evaluate the relationship between clinical and pathological disease in R-EAE, we correlated pathology scores (Table 5) with the severity of the initial clinical attack and any subsequent relapse (Table 4) in individual mice. Regression analyses indicated a moderate but statistically significant correlation between relapse severity and both demyelination ($r = 0.64$; $P > 0.6$). These results suggest that in addition to preventing demyelination and meningeal inflammation, the overall clinical benefits of SCH94.03 were secondary to inhibition of disease processes not readily identifiable by standard pathological analysis.

Table 4. Clinical Disease in SJL/L Mice With R-EAE After Treatment With SCH94.03

	TREATMENT	
	SCH94.03	Control*
Number of Mice	14	19
Initial attack		
Peak (day)	13 ± 1‡	14 ± 1
Maximal clinical severity	2.8 ± 0.2‡	2.8 ± 0.2
First relapse		
No. mice relapsed (%)	5/14 (35.7)§	15/19 (78.9)
Onset¶	2.4 ± 2 **	18 ± 2
Maximal clinical severity	2.4 ± 2 ‡	2.1 ± 0.2
Second Relapse		
No. mice relapsed (%)	0/14(0.0)‡‡	4/19 (21.1)
Onset¶	-	29 ± 2
Maximal clinical severity	-	2.3 ± 0.4
Cumulative relapses	4	19
Length of follow-up (days)	56 ± 1 ‡	58 ± 1

SJL/J mice with R-EAE were injected with 50 μg SCH94.03, IgM, or an equivalent volume of PBS twice weekly after spontaneous recovery from the initial episode of clinical disease. Subsequent relapses were assessed and graded

for severity. the data are a composite of 4 independent experiments and are presented as the means ± SEM where appropriate.

* Combined data from mice treated with control IgM (*n* = 10) or PBS (*n* = 9). No differences were observed with any disease parameter between the two control groups.

‡ Not significant ($P > 0.05$) when compared to control data using a Mann-Whitney rank sum test.

§ $P < 0.03$ when compared to control data using a Fisher exact test.

¶ Number of days from the peak of the initial attack.

** $P < 0.05$ when compared to control data using a Mann-Whitney rank sum test.

‡‡ $P = 0.12$ when compared to control data using a Fisher exact test.

Table 5. *Pathological Disease in SJL/J Mice with R-EAE After Treatment with SCH 94.03*

	Pathological Score		
Treatment	*n*	Demyelination	Meningeal inflammation
SCH94.03	14	24.6 ± 3.6*	18.7 ± 3.6*
Control ‡	19	39.3 ± 6.0	31.8 ± 5.3

SJL/J mice with R-EAE were treated as described in the Table 1 legend. The pathological scores were determined by a semi-quantitative morphological analysis and represent the percentage of spinal cord quadrants with the indicated pathological abnormality. One mouse treated with control IgM had minimal gray matter inflammation, whereas all other animals shoed no inflammation in spinal cord gray matter. The data are from 4 independent experimented and are presented as the mean ± SEM where *n*d indicates the number of mice.
*$P < 0.05$ when compared to control data.
‡ Combined data from mice treated with control IgM or PBS as described in the Table 4 legend.

Thus, it is reasonable to predict that autoantibodies, such as SCH94.03, play a critical role in stopping an immune-mediated process of demyelination in CNS diseases.

Two potential mechanisms can be proposed by which Abs promote remyelination. First, Abs might inhibit some pathogenic component of the disease process, such as virus activity, an immune response which directly suppresses remyelination. If the disease outcome is based upon a balance between tissue destruction and repair, inhibition of pathogenic components would allow a physiological repair response to predominate. Experimental and clinical evidence support this hypothesis. Spontaneous CNS remyelination is seen in MS patients and several experimental models of CNS demyelination as well as described herein, demonstrating spontaneous remyelination in control mice. This indicates that remyelination is a normal physiological response to myelin damage. In addition, treatment of mice chronically infected with TMEV with various immunosuppressive regiments promotes remyelination, but does not decrease demyelination, indicating that there is an immunological component which inhibits remyelination. Immunological function studies reported in Miller et al., International Immunology, (1996) 8, pp 131-141, The disclosure of which is incorporated herein by reference, indicate that animals treated with SCH94.03 had similar numbers of B and T (both CD4+ and CD8+) cells in their spleens compared to control animals, had similar *in vitro* splenocyte proliferative responses to mitogens and antigens, and mounted comparable Ab responses to both T cell-dependent and T cell-independent antigens. See Table 6, below. However, there was a 2 to 3 fold reduction in the number of CD4 and CD8 T cells infiltrating the CNS of mice treated with the mAb 94.03. Treatment with 94.03 also suppresses the humoral immune

response to a T cell-dependent antigen in chronically infected mice. Immuhistochemical staining showed that 94.03 labeled MHC Class II positive dendrite cells in peripheral lymphoid organs. These results thus suggest that one of the mechanisms by which Mab SCH94.03 maybe promoting remyelination is by inhibiting a pathogenic immune response.

Table 6. FCM analysis of mononuclear cells infiltrating the CNS of chronically infected SJL/J mice.

Treatment	N	Total n. of surface marker positive CNS-infiltrating mononuclear cells (x 10^{-5})[a]			
		$CD5^+$	$CD4^+$	$CD8^+$	CD45R(B220)
PBS	10	6.2 ± 0.8	3.0 ± 0.4	2.4 ± 0.3	0.4 ± 0.1
Control IgM	12	5.0 ± 0.6	3.0 ± 0.4	1.7 ± 0.2	0.2 ± 0.0
SCH94.03	12	2.3 ± 0.4[b]	1.4 ± 0.2[c]	0.8 ± 0.2[b]	0.1 ± 0.0[d]

SJL/J mice chronically infected with TMEV were injected i.p. with a total dose of 0.5 mg SCH94.03, control IfgM or an equivalent volume PBS, divided into twice weekly doses for 5 weeks. For control IgM, MOPC104E and XXMEN-OE5 were used. The data are a composite of independent experiments and are presented as the mean ± SEM, where *N* indicates the number of mice.

[a]Cell numbers were calculated by multiplying the percentage of positive cells assessed by FCM with the total number of mononuclear cells isolated from brain and spinal homogenates of individual mice by Percoll gradient separation.

[b]$P<0.00001$ when compared with combined control IgM and PBS data.

[c]$P<0.00005$ when compared with combined control IgM and PBS data.

[d]$P<0.007$ when compared with combined control IgM and PBS data.

The second hypothesis is that certain Abs can actively stimulate CNS remyelination, perhaps via stimulation of oligodendrocyte proliferation and/or differentiation *in vivo*, as has been demonstrated *in vitro* (Diaz, M. *et al.*, Brain Res., 154:231-239 (1978); Raine, C.S., et al., Lab. Invest., 38:397-403 (1979); Lehrer, G.M. et al., Brain Res., 172:557-560 (1979); Bansal, R. et al., J. Neurosci. Res., 21:260-267 (1988); Benjamins, J.A. and Dyer, C.A., Ann. NY Acad. Sci., 605:90-100 (1990); Dyer, C.A., Mol. Neurobiol., 7:1-22 (1993)). MAb SCH94.03 may directly stimulate precursor glial cells which are known to be present at the edges of both human and experimental CNS lesions which show active remyelination. Alternatively, SCH94.03 may work indirectly, via activation of astrocytes or other accessory cells, which could release factors important for the survival or proliferation of cells in the oligodendroglial lineage. The formation of Ab-antigen complexes *in situ* with tissue components released upon myelin destruction may also participate in Ab-mediated CNS remyelination. Although SCH94.03 is not CNS-specific, the recognition of both surface and cytoplasmic antigens on glial cells by the mAb supports an active mechanism hypothesis. In contrast to the immunomodulatory hypothesis, which would not necessarily require that Abs has direct access to the CNS, the hypothesis that Abs actively stimulate CNS remyelination implies the prerequisite of direct access to the CNS. This is contrary to the view of the selective permeability of the blood-brain barrier, especially toward large molecules such as pentameric IgM. However, during chronic inflammatory conditions such as TMEV infection or MS, peripheral leukocytes migrate into the CNS, indicating an alteration in the blood-brain barrier permeability. Therefore, large proteins such as serum Ig might also enter, via either passive diffusion through

"open" endothelium, or perhaps via an unidentified active transport mechanism.

Treatment of Demyelinating Diseases

The results of the experiments described herein have practical applications to multiple sclerosis (MS), EAE, and other related central nervous system demyelinating disorders. Rare examples of spontaneous CNS-type remyelination ("shadow plaques") are found in MS and occasional peripheral nervous system (PNS)-type remyelination is found in demyelinated spinal cord plaques near the root entry zone. Oligodendrocytes are infrequent at the center of the chronic plaques in MS but they appear to proliferate at the periphery of plaques, where they are associated with abortive remyelination. The process of remyelination may correlate with the spontaneous remission and improvements observed clinically in MS. These clinical observations indicate that new myelin formation is possible in MS. The remyelination that has been stimulated in mice with TMEV-induced demyelination by using a mAb holds promise for therapeutic applications in multiple sclerosis.

Of importance clinically is the question of whether morphologic regeneration of thin myelin sheaths contributes to functional recovery. Computer simulations indicate that new myelin formation even by inappropriately thin sheaths improves impulse conduction. Since the axon membrane of normally myelinated fibers is highly differentiated, it is necessary for sodium channels to be present at high density at the node of Ranvier to propagate saltatory conduction. Experimental evidence suggests that newly formed nodes do develop the required high sodium channel density as demonstrated by saxitoxin binding. Data to date suggest that remyelination even by inappropriately thin myelin improves conduction in a previously demyelinated axon.

Therefore, any strategy to promote this morphologic phenomenon has the potential of producing functional recovery.

The data presented herein demonstrates, for the first time, that administration of a monoclonal antibody to a mammal is capable of stimulating remyelination of central nervous system axons *in vivo*. Specifically, treatment of chronically infected TMEV-infected mice with as little as 10 μg of SCH94.03 resulted in a 4- to 5-fold increase in the total area of CNS myelination compared to mice treated with a control mAb.

Thus, as a result of the experiments described herein, the method of the present invention can be used to treat mammals, including humans and domestic animals, afflicted with demyelinating disorders, and to stimulate remyelination of the CNS axons. As described herein, an effective amount of the monoclonal antibody can be administered by conventional routes of administration, and particularly by, intravenous (iv) or intraperitoneal (ip) injection. An effective amount of the antibody can vary depending on the size of the mammal being treated, the severity of the disease, the route of administration, and the course of treatment. For example, each dose of mAb administered can range from approximately 0.5 mg/kg to approximately 400 mg/kg, with the preferred range from approximately 0.5 mg/kg to approximately 250 mg/kg. It is important to note that a dose as low as 10 μg (0.5 mg/kg) was effective in promoting remyelination of CNS axons in mice. The dose of mAb will also depend on the route of administration. For example, an iv dose administered to mice was 0.5 mg/kg, and an ip dose was 5.0 mg/kg. The course of treatment includes the frequency of administration of the mAb (e.g., daily, weekly, or bi-weekly) and the duration of the treatment (e.g., four weeks to four months). Thus, for example, a

larger amount of mAb can be given daily for four to five weeks, as opposed to a smaller amount of mAb given for four months.

The effectiveness of the amount of the monoclonal antibody being administered can be assessed using any number of clinical criteria, for example, as described in Example 3, including overall appearance of the mammal, the activity of the mammal and the extent of paralysis of the mammal. The effectiveness of the amount of monoclonal antibody necessary to induce remyelination in humans can also be assessed in a double blinded controlled trial. Patients with fixed neurological deficits from demyelinating disease can be treated with monoclonal antibody or controls. Improvement in isometric muscle strength as detected by quantitative biomechanics muscle testing could be used as the primary therapeutic end-point.

Additionally, the monoclonal antibody may be genetically altered, e.g. "humanized" by the substitution of human antibody nucleotide sequences in non-variable regions of the murine mAb to reduce immunogenicity.

In addition to *in vivo* methods of promoting remyelination, *ex vivo* methods of stimulating remyelination in CNS axons are also encompassed by the present invention. For example, the monoclonal antibody may be used *in vitro* to stimulate the proliferation and/or differentiation of glial cells, such as oligodendrocytes, as described in Example 2. These exogenous glial cells can then be introduced into the CNS of mammals using known techniques. Remyelination of CNS axons would be increased by increasing the number of endogenous glial cells present (glial cells, such as oligodendrocytes play a critical role in the production of myelin).

In vitro methods of producing glial cells, or stimulating the proliferation of glial cells from mixed culture (e.g., rat optic nerve cell, or rat brain cell cultures) are also encompassed by this invention. For example, cells obtained from rat optic nerve, or rat brain, containing glial cells, are cultured as a mixed culture under conditions sufficient to promote growth of the cells. An effective amount of mAb capable of promoting remyelination of CNS axons, such as SCH94.03, is then added to the mixed culture of cells and maintained under conditions sufficient for growth and proliferation of cells. The mAb stimulates the proliferation of glial cells cultured in the presence of the mAb is increased, relative to the proliferation of glial cells grown in the absence of the mAb.

The monoclonal antibodies for use in the methods of the present invention can be, and are preferably, administered as medicaments, i.e., pharmaceutical compositions. An effective amount of the monoclonal antibody can thus be combined with, or diluted with, an appropriate pharmaceutically acceptable carrier, such as a physiological buffer, or saline solution.

The pharmaceutical compositions used in the methods of this invention for administration to animals and humans comprise the monoclonal antibodies in combination with a pharmaceutical carrier or excipient.

The medicament can be in the form of tablets (including lozenges and granules), dragees, capsules, pills, ampoules or suppositories comprising the compound of the invention.

Advantageously, the compositions are formulated as dosage units, each unit being adapted to supply a fixed dose of active ingredients. Tablets, coated tablets, capsules,

ampoules and suppositories are examples of preferred dosage forms according to the invention. It is only necessary that the active ingredient constitute an effective amount, i.e., such that a suitable effective dosage will be consistent with the dosage form employed in single or multiple unit doses. The exact individual dosages, as well as daily dosages, will, of course, be determined according to standard medical principles under the direction of a physician or veterinarian.

The monoclonal antibodies can also be administered as suspensions, solutions and emulsions of the active compound in aqueous or non-aqueous diluents, syrups, granulates or powders.

Diluents that can be used in pharmaceutical compositions (e.g., granulates) containing the active compound adapted to be formed into tablets, dragees, capsules and pills include the following: (a) fillers and extenders, e.g., starch, sugars, mannitol and silicic acid; (b) binding agents, e.g., carboxymethyl cellulose and other cellulose derivatives, alginates, gelatine and polyvinyl pyrrolidone; (c) moisturizing agents, e.g., glycerol; (d) disintegrating agents, e.g., agar-agar, calcium carbonate and sodium bicarbonate; (e) agents for retarding dissolution, e.g., paraffin; (f) resorption accelerators, e.g., quaternary ammonium compounds; (g) surface active agents, e.g., cetyl alcohol, glycerol monostearate; (g) adsorptive carriers, e.g., kaolin and bentonite; (i) lubricants, e.g., talc, calcium and magnesium stearate and solid polyethylene glycols.

The tablets, dragees, capsules and pills comprising the active compound can have the customary coatings, envelopes and protective matrices, which may contain opacifiers. They can be so constituted that they release the active ingredient only or preferably in a particular

part of the intestinal tract, possibly over a period of time. The coatings, envelopes and protective matrices may be made, for example, from polymeric substances or waxes.

The diluents to be used in pharmaceutical compositions adapted to be formed into suppositories can, for example, be the usual water-soluble diluents, such as polyethylene glycols and fats (e.g., cocoa oil and high esters, [e.g., C_{14}-alcohol with C_{16}-fatty acid]) or mixtures of these diluents.

The pharmaceutical compositions which are solutions and emulsions can, for example, contain the customary diluents (with, of course, the above-mentioned exclusion of solvents having a molecular weight below 200, except in the presence of a surface-active agent), such as solvents, dissolving agents and emulsifiers. Specific non-limiting examples of such diluents are water, ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (for example, ground nut oil, glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitol or mixtures thereof.

For parental administration, solutions and suspensions should be sterile, e.g., water or arachis oil contained in ampoules and, if appropriate, blood-isotonic.

The pharmaceutical compositions which are suspensions can contain the usual diluents, such as liquid diluents, e.g., water, ethyl alcohol, propylene glycol, surface active agents (e.g., ethoxylated isostearyl alcohols, polyoxyethylene sorbitols and sorbitan esters), microcrystalline cellulose, aluminum methahydroxide, bentonite, agar-agar and tragacanth, or mixtures thereof.

The pharmaceutical compositions can also contain coloring agents and preservatives, as well as perfumes and flavoring additions (e.g., peppermint oil and eucalyptus oil), and sweetening agents, (e.g., saccharin and aspartame).

The pharmaceutical compositions will generally contain from 0.5 to 90% of the active ingredient by weight of the total composition.

In addition to the monoclonal antibodies, the pharmaceutical compositions and medicaments can also contain other pharmaceutically active compounds.

Any diluent in the medicaments of the present invention may be any of those mentioned above in relation to the pharmaceutical compositions. Such medicaments may include solvents of molecular weight less than 200 as the sole diluent.

It is envisaged that the monoclonal antibodies will be administered perorally, parenterally (for example, intramuscularly, intraperitoneally, subcutaneously, transdermally or intravenously), rectally or locally, preferably orally or parenterally, especially perlingually, or intravenously.

The administered dosage rate will be a function of the nature and body weight of the human or animal subject to be treated, the individual reaction of this subject to the treatment, type of formulation in which the active ingredient is administered, the mode in which the administration is carried out and the point in the progress of the disease or interval at which it is to be administered. Thus, it may in some case suffice to use less than a minimum dosage rate, while other cases an upper limit must be exceeded to achieve the desired

results. Where larger amounts are administered, it may be advisable to divide these into several individual administrations over the course of the day.

The present invention will be better understood from a consideration of the following examples, which describe the preparation of compounds and compositions illustrative of the present invention. It will be apparent to those skilled in the art that many modifications, both of materials and methods, may be practiced without departing from the purpose and intent of this disclosure.

EXAMPLES

Example 1

Monoclonal Antibody Production, Screening and Purification

Animals

Spleens of two SJL/J mice (Jackson Laboratories, Bar Harbor, ME) that had been injected twice with spinal cord homogenate (SCH) in incomplete Freund's adjuvant were used as the source of B cells for fusion and hybridoma production. Splenocytes were fused with NS-1 myeloma cells using polyethylene glycol, and viable cell fusions were selected with hypoxanthine-aminopterin-thymidine (HAT) media and cloned by limiting dilution as described (Katzmann, J.A. et al., Proc. Nat. Acad. Sci. USA, 78:162-166 (1981)).

ELISAs

Hybridoma supernatants from viable Ig-producing clones were screened for binding to SCH by an enzyme-linked immunosorbant assay (ELISA). The following antigens were used for screening mAbs: SCH - (10 μg) reconstituted in carbonate-bicarbonate buffer (pH 8.53), MBP - (1 μg) dissolved in PBS, GC (1 μg) dissolved in absolute alcohol, PLP (1 μg) dissolved in water. PLP was provided by Dr. W. Macklin (UCLS) who has published a solid phase immunoassay for PLP. For SCH, MBP or GC ELISA, Immuno II plates were coated with prepared antigen (100 μl/well) which was incubated overnight at 4°C. The following day well were washed in PBS and blocked with PBS + 1% serum for 1 hour at room temperature. Plates were washed again in PBS and serial dilutions of primary Ab diluted in PBS/0.1% BSA were added and incubated at room temperature for 2 hours. Plates were washed in PBS/0.05% Tween and appropriate secondary Ab conjugated to alkaline phosphatase (1:1000 in PBS 0.1% BSA) was added. Plates were incubated at 37°C for 2 hours, washed in PBS 0.05%

Tween, and the substrate (Sigma 104 Phosphatase Substrate Tablet in 5 ml diethanolamine buffer) was added for 30 min. The reaction was terminated with 50 μl of 1 N NaOH. The plates were read on a Dynatech ELISA plate reader.

Ascites production

The hybridomas chosen for treatment experiments were injected into pristane-treated BALB/c mice for ascites production. Hybridomas were also grown in RPM1-1640 media supplemented with 10% fetal bovine serum for IgM production. IgM mAbs were purified by either ammonium sulfate precipitation and gel filtration on a Sephacryl S-400 HR (Sigma) column for the initial transfer experiments, or by affinity chromatography using goat anti-mouse IgM (μ-chain specific; Jackson Immunoresearch, West Grove, PA) coupled to Reacti-Gel 6X matrix (Pierce, Rockford, IL) for later transfer experiments.

Example 2

In Vitro Testing of Monoclonal Antibodies

Selection of mAbs that promote glial cell proliferation

The ability of the mAbs to promote proliferation of glial cells *in vitro* was tested. Glial cells isolated from rat brain or optic nerves were seeded in Falcon Microtest II plates at a concentration of 2 x 10_4 cells per well in 0.1 ml of DME. Whole serum (SCH, IFA, MBP, GC, MBP/GC, PBS or PLP), purified Ig or mAb, was serially diluted and 0.1 ml aliquot was added to cells and assayed in triplicate. Three days later ^{3}H-thymidine was added (1 μCi/ml) and cells were harvested after 17 hours with an automated cell harvester (Mash II Harvester). To document identity of cells proliferating (i.e., astrocytes, progenitor glial cells, macrophages), selected cultures after exposure to ^{3}H-thymidine, were incubated with appropriate Ab specific for cell type followed by ABC immunoperoxidase technique. After reaction of Hanker-Yates reagent, the slides were immersed in Ilford K2

nuclear emulsions, exposed for 4 days at 4°C and developed.

<u>mAb 94.03 and 94.32 induce proliferation of mixed rat optic nerve brain cultures</u>

One- to two-day-old rats were killed with ether. Through careful dissection, optic nerves were removed from the optic nerve chiasm to the eye. Nerves were transferred to centrifuge tubes containing 2 mls of DMEM. An equal volume of 0.25% trypsin was added and incubated to 37°C in a water bath for 45 min. 0.2 ml of FCS was added to terminate trypsinization. Nerves were passed through a sterile needle and syringe (gauge no. 21) and then centrifuged at 1400 rpm for 10 minutes. The cell count was adjusted to provide concentration of 5 x 10^5 cells/100 µl of media in 24-well trays in DMEM + 0.5% FCS. After 12 to 16 hours, appropriate antibodies or growth media were added as per experimental protocols.

Brains of 1-2 day old rats were removed and placed in Hank's Balanced Salt Solution with 10 mM HEPES buffer (HBSS/H), approximately 1-2 ml per brain. The brain stem, cerebellum, nd midbrain was discarded whereas the forebrain was minced with a bent syringe. The tissue was further disrupted by repeated passage through a 10 ml pipet and transferred to a 50 ml conical tube. The tissue suspension was shaken on a rotary shaker (75 rpm) for 30 min at 37°C. Trypsin was added to a final concentration of 0.125% and the suspension was shaken for an additional 60 minutes. Trypsin digestion was stopped by adding FCS (10%). The cell suspension was passed sequentially through 120 and 54 µm Nytex, centrifuged, resuspended in serum-free medium with 10% FCS, and filtered again through 54 µm Nytex. Serum-free media was DMEM with 3.7 g/l sodium bicarbonate, 6.0 g/l glucose, 2 mM L-glutamine, 0.1 nM nonessential amino acids, 5 µg/ml insulin, 5 µg/ml streptomycin. The cells were counted, plated onto uncoated tissue culture flasks or plates at

$5x10_4$ cells/cm_2 and cultured at 37°C in 5% CO_2. The media was changed after 72 hours, and every 48 hours thereafter. On day 8 after culture initiation, the media was aspirated and replaced by SFM with various supplements (for example, antibody). For most experiments, the cells were grown for an additional 48 hours before harvesting. Cells were pulsed with [^{3}H]thymidine (5 μCi/ml) for the final 1824 hours of culture.

Western Blot Procedure

Antigens were denatured and solubilized by heating at 100°C in sodium dodecyl sulfate (SDS) sample buffer. Samples were electrophoresed on stacking and separating gels containing 4.75% and 12.0% acrylamide at 200 volts. After electrophoresis, gels and nitrocellulose membranes were equilibrated for 30 minutes in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.1-8.3). All steps were done at room temperature. Gels were electroblotted for either 1 hour at 100V or overnight at 30V using the Bio-Rad Mini Trans-blot apparatus. The nitrocellulose membrane was cut into strips and washed, 3X TBS (100 mM NaCI, 50 mM Trig, pH 7.6) with 0.03% Tween 20. Nitrocellulose strips were blocked (TBS with 3% non-fat milk and 0.03% Tween 20) for 2-4 hours, washed 3X, and incubated with primary Ab or antisera (diluted in blocking buffer) for 4 hours or overnight. After primary Ab incubation, strips were washed 3X, incubated with either biotin- or alkaline phosphate-labelled secondary Ab (diluted in blocking buffer) for 2 hours, washed 3X, and incubated with alkaline-phosphatase labeled-streptavidin (diluted in blocking buffer) for 2 hours if the biotin system is used. Nitrocellulose strips were washed 4X (final wash in TBS without Tween 20) and incubated with substrate solution (0.165 mg/ml BCIP and 0.33 mg/ml NBT in 100 mM sodium chloride, 100 mM Tris, 5 mM MgG12, pH 9.5) until sufficient color developed

(approximately 10-15 min). The reaction was stopped by adding PBS with 5 mM EDTA.

Cell lines or mixed brain cultures were lysed in 1X SDS reducing sample buffer (2.3% SDS, 10% 2-ME, 0.125 M Tris, 20% glycerol) and heated to 85°C for 15 minutes. Nucleic acids were sheared by repeated passage of lysate through 21-27-gauge needles. Lysate proteins were separated on a 12% acrylamide reducing gel, transferred to nitrocellulose membranes, and blotted with various antibodies as previously described.

Example 3

Promotion of CNS Remyelination Using a Monoclonal Antibody

Virus

The DA strain of TMEV was obtained from Drs. J. Lehrich and B. Arnason after eight passages in BHK cells. The virus was passaged an additional four times at a multiplicity of infection of 0.1 plaque forming units (PFU) per cell. Cell-associated virus was released by Freeze-thawing the cultures followed by sonication. The lysate was clarified by centrifugation and stored in aliquots at -70°C. All subsequent experiments will use passage 12 virus. This virus isolate causes white matter pathology without destruction of anterior horn cells.

In vitro TMEV neutralization assay

Viral plaque assays were done as previously described (Patick, A.K., et al., J. Neuropath. Exp. Neurol., 50:523-537 (1991)). To assess neutralization, aliquots of TMEV (200 PFU/ml) were incubated with various concentrations of Ab for 1 hour t room temperature prior to plating onto confluent L2 cells. As a positive control, serum from susceptible mice chronically infected with TMEV was used. Under the assay conditions described above, a serum dilution of 1:34,000 gave 50%

neutralization, which corresponded to an estimated 20 ng/ml of TMEV-specific Abs, assuming a total serum Ig concentration of 15 mg/ml, and a TMEV-specific fraction of 5%.

Demyelination protocol

Demyelination was induced in female SJL/J mice, ages four to six weeks, from the Jackson Laboratory, Bar Harbor, ME. Mice were inoculated intracerebrally with 2 x 10^5 plaque-forming units of DA virus in a volume of 10 μl. Mice infected chronically with TMEV (4 to 6 months following infection) were assigned randomly to groups of treatment.

Treatment protocol and clinical disease assessment

Chronically infected mice were given either intraperitoneal (ip) or intravenous (iv) injections of mAb twice weekly for 4-5 weeks. At each treatment injection, mice were assessed clinically by three criteria: appearance, activity, and paralysis. A score for each criterion was given ranging from 0 (no disease) to 3 (severe disease). For appearance, 1 indicated minimal change in coat, 2 indicated a severe change (incontinence and stained coat). For activity, 1 indicated decreased spontaneous movements (minimal ataxia), 2 indicated moderate slowing (minimal spontaneous movements), and 3 indicated severe slowing (no spontaneous movement). For paralysis, 0.5 indicated a spastic extremity, 1 indicated a paralyzed extremity, 1.5 indicated two or more spastic extremities, 2 indicated two paralyzed extremities (unable to walk), 2.5 indicated no righting response, and 3 indicated three or four paralyzed extremities (moribund). The total score for each mouse was the cumulative total from each criterion (maximum of 9). As the clinical score was an ordinal, but not a cardinal scale, the change in clinical score to assess clinical disease was used. The clinical

assessment data were not disclosed until after the morphological assessment of remyelination was completed.

Light and electron micrograph preparation and assessment of remyelination

Preparation of light and electron microscopy sections and morphological assessment of remyelination were done. Briefly, treated mice were anesthetized with pentobarbital (0.2 mg ip), exsanguinated by cardiac puncture, and filled by intracardiac perfusing with Trump's fixative (100 mM phosphate buffer, pH 7.2, with 4% formaldehyde and 1.5% glutaraldehyde). The entire spinal cord was removed carefully from the spinal canal, and sectioned into 1 mm transverse blocks. Every third block was post-fixed in 1% osmium tetroxide and embedded in Araldite (Polysciences, Warrington, PA). One micron sections from each block were cut and stained with p-phenylenediamine. On each section, remyelination was quantitated using a Zeiss interactive digital analysis system (ZIDAS) and camera lucida attached to a Zeiss photomicroscope (Carl Zeiss Inc., Thornwood, NY). Abnormally thin myelin sheaths relative to axonal diameter was used as the criterion for CNS remyelination. Ten spinal cord sections from each mouse were examined; this corresponded to 8-9 mm^2 of white matter examined per mouse. To avoid bias, slides were coded and quantitation was done without knowledge of the treatment groups.

Myelin thickness and axonal diameter measurements and quantitation of myelination axons

Electron micrographs of normal and remyelinated axons from plastic-embedded spinal cord sections were imaged with a Hamamatsu video camera, digitized, and analyzed using an IBAS 2000 Image Analysis System (Kontron, Munich, Germany). The Axonal cross-sectional area with and without the myelin sheath was measured, and equivalent circle calculations were used to determine the

axonal diameter and myelin sheath thickness. For myelinated axon quantitation, the number of myelinated axons in lesions from plastic-embedded spinal cord sections were counted using the analysis system described above attached to an Axiophot microscope (Carl Zeiss, Inc.). 17 remyelinated and 15 demyelinated lesions in spinal cord sections from animals treated with mAb SCH94.03, control IgM, or buffer only were analyzed. This corresponded to 0.6mm^2 of remyelinated area and 0.8 mm^2 of demyelinated area. The criterion for selection of a lesion as demyelinated was the presence of substantial demyelination with minimal repair, whereas remyelinated lesions were chosen based upon the presence of almost complete remyelination throughout the lesion.

<u>Immunostaining</u>

Rat 5.5B8 glial cells were grown on poly-D/L-lysine-coated chamber slides in Dulbecco's modified Eagle's medium (DMED) supplemented with 1.5 g/L D-glucose, 30 nM SeO_2, 15 nM triiodothyronine, 10 ng/ml biotin, 100 µM $ZnCl_2$ 50 µg/ml gentamicin, and 10% fetal bovine serum. All staining steps were done at room temperature. For surface staining, slides were briefly rinsed with PBS, and cells were lightly fixed with 1% formaldehyde in PBS for 10 minutes to prevent cell detachment during subsequent staining steps. For cytoplasmic staining, slides were rinsed twice in PBS and either air dried for 1 hour or incubated with 0.1% Triton X-100 in PBS for 10 minutes. Cells were blocked in 2% BSA for 30 min, washed, incubated with control IgM or mAb SCH94.03 (10 µg/ml in 1% BSA) for 1 hour, and washed extensively with PBS. After fixation with 4% paraformaldehyde for 15 min, slides were incubated with fluorescein-labeled goat anti-mouse IgM (Jackson Immunoresearch) for 1 hour, washed with PBS, coverslipped with 10% MOWIOL® (Hoechst) in 100 mM Tris, 25% glycerol, pH 8.5 with 25 µg/ml 1,4-diazobicyclo-[2.2.2]-octane (DABCO) to prevent fading,

and allowed to set overnight in the dark. For frozen tissue sections, fresh neonatal rat, adult mouse, or autopsied human cortical brain tissue was quick frozen in isopentane chilled in liquid nitrogen prior to liquid nitrogen storage. Frozen sections (10 μm) were transferred onto gelatinized glass microscope slides, air dried for 4-8 hours, and stored at -70°C. Prior to immunostaining, slides were placed at room temperature overnight. The immunoperoxidase staining protocol was similar that described above, using the ABC immunoperoxidase reagent (Vector Laboratories, Burlingame, CA), developed with 1.5 mg/ml Hanker-Yates reagent (p-phenylene diamine-procatechol) in 50 mM Tris, pH 7.6 with 0.034% H2O2, counterstained with Mayer's hematoxylin, and mounted with Permount (Fischer Scientific, Pittsburgh, PA).

Data Analysis

A modified cumulative rank sum test (O'Brien, P.C., Biometrics, 40:1079-1087 (1984)) was used to compare remyelination between treatment groups. This statistical test takes into account several numerically unrelated parameters of therapeutic effectiveness, and is used routinely for clinical trial efficacy assessment. Parallel analyses using a standard unpaired Student's t-test to compare individual parameters of remyelination gave equivalent results. Comparisons of disease severity and correlation significance were determined by a one-way analysis of variance (ANOVA). Statistical analyses were done with either the SigmaStat (Jandel Scientific, San Rafael, CA) or EXCEL (Microsoft Corporation, Redmond, WA) software programs. Calculated values were considered significant when p was < 0.05.

Example 4

1. Hybridoma culture and determination of Ig isotype

A2B5, HNK-1, and XXMEN-OE5 (anti-bacterial lipopolysaccharide) hybridomas were purchased from American Type Culture Collection (Rockville, MD). O1 and O4 hybridomas were the gift of Dr. S.E. Pfeiffer (University of Connecticut, Farmington, CT). Hybridomas were cultured in RPMI 1640 containing 10% fetal calf serum (HyClone, Logan, UT) and 2 x 10^{-2} mM ß-mercaptoethanol. IgM concentrations of the supernatants were determined by a μ-chain-specific capture ELISA With purified MOPC104E (Sigma, St. Louis, MO) as the standard. To determine the IgM isotype of mAbs O1, O4, and A2B5, Mouse Monoclonal Antibody Isotyping Kit (Gibco, Grand Island, NY) was used.

2. mRNA isolation and cloning of Ig variable region

Poly(A)$^+$ RNA was isolated from hybridoma cells by oligo(Dt)-cellulose chromatography using the Micro-Fast Track kit (Invitrogen, San Diego, CA). Ig heavy and light chain variable region cDNAs were cloned by the 5′-rapid amplification of cDNA ends (RACE) method using the 5′-AmpliFINDER™ RACE kit (Clontech, Palo Alto, CA). Briefly, first strand cDNA was synthesized using an oligo Dt primer. An anchor oligonucleotide was ligated to the 3′ end of the first strand cDNA, and variable region cDNAs were amplified by polymerase chain reaction using primers corresponding to the anchor sequence and constant region-specific primers for the μ ($C\mu$) or $_\kappa$ (C_κ) chains described previously [Miller et al., J. Immunol., 154 (1995), 2460-2469].

3. Sequencing and analysis

Amplified cDNA products were purified from agarose gel after electrophoresis and directly subcloned into pCRII using the TA cloning kit (Invitrogen). Both strands of the insert were sequenced using automated DNA sequencer (Applied Biosystems model 373A, Mayo Molecular Biology Core Facility). For nucleotide sequence homology

searches, the FastA program (GCG program, version 8) was used [Devereux, J. et al., Nucleic Acids Res., 12 (1984), 387-395].

4. Direct ELISA to determine polyreactivity

HNK-1 was shown previously to be polyreactive by Western blots [McGarry et al., *supra*]. Therefore, the polyreactivity of O1, O4 and A2B5 was tested by direct ELISA. Human RBC spectrin, bovine myosin (heavy chain), mouse albumin, mouse hemoglobin, mouse transferrin, hen egg lysozyme, rabbit actin, rabbit myelin basic protein, and keyhole limpet hemocyanin (KLH) were purchased from Sigma. Proteins were tested for purity by SDS-polyacrylamide gel electrophoresis. The chemical hapten trinitrophenyl (TNP) was coupled to bovine serum albumin (BSA) [Miller et al., 1995, *supra*]. Protein antigens were used at 5 μg/ml, and hapten was used at 2 μM. The proteins and hapten-BSA antigens were coated onto polystyrene or polyvinylchloride microtiter plates in 0.1 M carbonate buffer, pH 9.5, for 18 hours at 4°C. Coated plates were blocked with PBS containing 5% nonfat dry mild and 0.05% Tween 20 for 2 hours at room temperature, and incubated with mAbs diluted in blocking buffer (2 μg/ml) for 4 hours at room temperature. TEPC183 (Sigma) and XXMEN-OE5 IgM mAbs were used as control antibodies. Bound IgM was detected with biotinylated goat anti-mouse IgM (μ chain specific; Jackson Immunoresearch, West Grove, PA) followed by alkaline phosphatase conjugated to streptavidin, with p-nitrophenylphosphate as the chromogenic substrate. Absorbance was determined at 405 nm.

Results

Nucleotide sequences of variable region cDNA including leader peptide were compared with published sequences of germline genes, mouse myeloma and natural autoantibodies.

1. Heavy chain variable region cDNA sequences

O1 V_H was identical with unrearranged V_H segment transcripts A1 and A4 [Yancopoulos et al. Cell, 40 (1985), 271-281], which belong to V_H558 family (Figure 12). The O1 D segment was relatively short and contained four nucleotides derived from germline SP2 gene family (common sequence to DSP2.3, 2.4 and 2.6) [Kurosawa et al., J. Exp. Med., 155 (1982), 201-218]. The D segment for O1 was dG and dC rich in the 5′ end, probably representing non-coded (N) nucleotides inserted by terminal deoxynucleotide transferase (TdT) during V-D-J recombination. O1 displayed sequence identity with germline J_H1 [Sakano et al., Nature, 286 (1980), 676-683], except for one nucleotide (GTC for GTT in the germline), which did not result in an amino acid substitution.

Compared with the germline BALB/c V_H101 [Kataoka et al., J. Biol. Chem., 257 (1982), 277-285], O4 V_H showed three nucleotide differences in the V_H coding region (Figure 13), all of which resulted in amino acid substitutions. Compared to natural autoantibody D23 [Baccala et al., Proc. Natl. Acad. Sci. USA, 86 (1989), 4624-4628], which is encoded by germline V_H101, O4, V_H showed two nucleotide differences with amino acid substitutions in the V_H coding region. Compared with germline V_H101, HNK-1 V_H showed six nucleotide differences and four amino acid differences in the V_H coding region (Figure 13). Compared to natural autoantibody D23, HNK-1 V_H showed five nucleotide differences and three amino acid differences in the V_H coding region. D23 had three nucleotide differences when compared with germline V_H101; all differences were also seen in the O4 and HNK-1 V_H. The O4 D segment contained five nucleotides and the HNK-1 D segment contained 13 nucleotides derived from germline DFL16.1 gene [Kurosawa et al., J. Exp. Med., 155 (1982),

201-218]. The HNK-1 D segment had one dG residue in the 5′ end and four dG residues in the 3′ end, which probably represent N nucleotides inserted by TdT during V-D-J recombination. The heavy chain joining region of O4 corresponded to germline J_H4 [Sakano et al., *supra*]. The heavy chain joining region of HNK-1 corresponded to germline J_H4 beginning with the fifth codon.

The A2B5 V_H showed seven nucleotide and four amino acid differences in its coding region in comparison with the germline V1 (also called T15 and S107) [Crews et al., Cell, 25 (1981), 59-66; Siu et al., J. Immunol., 138 (1987), 4466-4471] (Figure 14). The heavy chain joining region of A1B5 corresponded to germline J_H3 beginning with the third codon [Sakano et al., *supra*].

2. Light chain variable region cDNA sequences

Since all the hybridomas produced IgM $_\kappa$ antibodies as determined by isotyping assay, a C_κ primer was used for polymerase chain reaction. O1 and O4 light chain variable region cDNA sequences were identical (Figure 15). The V_κ segments of O1 and O4 were identical with natural autoantibody E7 [Baccala et al., *supra*], and showed only one silent nucleotide difference when compared with myeloma MOPC21 [Hamlyn et al., Nucleic Acids Res., 9 (1981), 4485-4494]. The J_κ segment of HNK-1 showed sequence identity with $J_\kappa2$.

The genomic germline gene which encodes the V_κ segment of A2B5 (Figure 17) is unknown, but belongs to the $V_\kappa19$ group [Potter et al., Mol. Immunol., 19 (1982), 1619-1630]. The V_κ segment of A2B5 was identical with the V_κ segment from hybridomas H220-11, H230-2, H230-5 and H250-6 [Caton et al., J. Immunol., 147 (1991), 1675-1686] except for two nucleotide changes, one of which resulted in an amino acid substitution (data not shown). The V_κ

segments of H220-11, H230-2, H230-5 and H250-6 are identical to each other. The J_x segment of A2B5 was identical with J_x5 [Max et al., J. Biol. Chem., 256 (1981), 5116-5120; Sakano et al., *supra*] except for one nucleotide which resulted in an amino acid substitution.

3. Direct ELISA

To assess the polyreactivity of the O1, O4, and A2B5, binding of mAbs to a panel of defined antigens was determined by ELISA (Figure 18). O1 reacted with human RBC spectrin. O4 reacted with human RBC spectrin, bovine myosin, mouse hemoglobin, rabbit actin, and TNP-BSA. A2B5 and the two control IgM_x mAbs did not react with this panel of antigens.

4. Utility

The enormous diversity in the Ig variable region is due primarily to combinations of multiple germline coding gene segments. Different primary structures are produced by recombination of V,D,J (heavy chain) or V,J (light chain) gene segments. Assuming the random association of heavy and light chains to form a complete antibody molecule, the number of different molecules is estimated to be 1.6×10^7 [Max et al., Fundamental Immunology, Raven Press, NY, 1993, pp. 315-382]. Somatic mutation during the process of antigen challenge provides even further diversity and specificity. In contrast to the majority of Igs produced following antigen challenge, natural autoantibodies are encoded directly by germline genes with no or few mutations. Natural autoantibodies are present in sera of healthy humans and rodents [Dighiero et al., J. Immunol., 131 (1983), 2267-2272; Guilbert et al., J. Immunol., 128 (1982), 2279-2287; Hartman et al., Mol. Immunol., 26 (1989), 359-370]. These natural autoantibodies are polyreactive, capable of binding to a variety of structurally unrelated antigens [Avrameas et al., Mol. Immunol., 30 (1993), 1133-1142].

The physiologic function of natural autoantibodies is unknown. However, by interacting with many self constituents, these natural autoantibodies and their targets are believed to establish a vast network whereby the immune system can participate in general homeostasis.

These results provide evidence based on Ig variable region cDNA sequences that three of the four OL-reactive IgM_κ mAbs (O1, O4 and HNK-1) have characteristics of natural autoantibodies. The J_H segments of O4 and HNK-1, and the J_κ segments of O1, O4 and HNK-1 are encoded by unmutated germline Ig genes. The J_κ segment of O1 has only one silent nucleotide change. O1 V_H is identical with unrearranged V*H* segment transcripts A1 and A4, which belong to the V_H558 family [Yancopoulos et al., *supra*]. Because the germline genes corresponding to the V_κ genes of myeloma MOPC21 V_κ19 gene family [Potter et al., *supra*] are unknown, direct evaluation of the somatic mutations of the light chains was not possible. However, O1 and O4 light chain variable regions are identical with the sequence reported for natural autoantibody E7 [Baccala et al., *supra*], and are identical with myeloma MOPC21 V_κ segment [Hamlyn et al., *supra*], except for one silent nucleotide change. This provides strong evidence that O1 and O4 V_κ segments are directly encoded by germline Ig genes. Though O4 and HNK-1 V had minor differences from germline V_H101 [Katsoka et al, J. Biol. Chem., 257 (1982) 277-285], their sequences are very close to D23 V_H sequence, a well-characterized natural autoantibody [Baccala et al., *supra*]. In addition, HNK-1 V_κ showed identity with myeloma MOPC41 [Seidman et al., Nature, 280 (1979), 370-375] and germline V_κ41 [Seidman et al., *supra*], except for two silent nucleotide changes. Our results were not able to determine whether A2B5 V_κ segment is encoded by germline Ig gene. However, the A2B5 V_κ is encoded by an unidentified germline Ig gene

rather than by extensive somatic mutation of a germline Ig gene.

The results also showed that O1 and O4 react to multiple different antigens as demonstrated by ELISA. This is consistent with the immunocytochemistry [Eisenbarth et al., *supra*; Sommer et al., *supra*] demonstrating the reactivity of these mAbs to intracellular antigens in many cells. HNK-1 was shown previously to be polyreactive by Western blots using the lysates of chick embryo spinal cord neuron-enriched cultures and rat brain [McGarry et al., *supra*].

The Ig cDNA sequences and polyreactivity to multiple antigens are consistent with the hypothesis that O1, O4 and HNK-1 are natural autoantibodies. In contrast, A2B5 does not show polyreactivity by ELISA and the Ig cDNA sequence similarity to the germline is undetermined. Characterization of O1, O4 and HNK-1 as natural autoantibodies raises the possibility that they exist normally in serum and have physiologic function during development or in CNS diseases. In support of a physiologic function for these mAbs is the report that O4 stimulates the differentiation of OLs *in vitro* [Bansal et al., *supra*]. Since Schwann cells share with OLs the antigens recognized by O1, O4 and HNK-1, this suggests that these mAbs may have a function not only in the CNS but also in the peripheral nervous system. Direct proof of this hypothesis awaits experiments with these mAbs *in vivo* during development and in animal models of CNS diseases.

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.